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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT:** *Our overall objective is to understand which tumor cell behaviors contribute to invasion and metastasis.* This would allow rationale approaches to limit these aspects of tumor progression. While great strides have defined critical molecular determinants, the current experimental models of tumor invasion limit the dissection of complex cellular responses. *In vitro* assays do not capture tumor/host relations or relevant tissue architecture and physiology. *In vivo* model systems provide the relevant organism contexts but cannot readily be manipulated. Quantal advances would be enabled by combining the best attributes – direct manipulation of tumor and host, long-term visualization, and tissue relevant architecture. Our central premise is that an ex vivo organotypic liver tissue system can provide an environment to study tumor cell invasion and metastasis. Our objective is to utilize a physiologically relevant microreactor that has proved suitable for organotypic liver culture to investigate metastatic seeding. The sub-millimeter scale of this liver allows for real-time imaging over weeks in culture. We established this system to determine what step is rate-limiting for tumor progression. We have now established an organotypic liver tissue culture that supports metastatic establishment and growth. This will be used to probe the molecular steps that are key to this progression.

**15. SUBJECT TERMS**
Tumor metastasis, novel technology, liver, bioreactor

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**AN ORGANOTYPIC LIVER SYSTEM FOR TUMOR PROGRESSION**

**Alan Wells, Linda Griffith, Donna Stolz, Douglas Lauffenburger**

## INTRODUCTION

*Our overall objective is to understand which tumor cell behaviors contribute to invasion and metastasis*. This would allow rationale approaches to limit these aspects of tumor progression. While great strides have defined critical molecular determinants, the current experimental models of tumor invasion limit the dissection of complex cellular responses. *In vitro* assays such as transmigration of barrier matrices or cell layers allow for targeted perturbations, but do not capture tumor/host relations or relevant tissue architecture and physiology (1). *In vivo* model systems, mainly xenografts and induced tumors, provide the relevant organism contexts but cannot readily be manipulated. Recent advances in imaging of tumors in living animals aids in documenting events, though these views still occur only over hours and only near the surface of observation (2). Thus, quantal advances would be enabled by new assay systems that combine the best attributes of both – direct manipulation of tumor and host, long-term (days to weeks) visualization, and tissue relevant architecture.

*Our central premise is that an ex vivo organotypic liver tissue system can provide an environment to study tumor cell invasion and metastasis*. Our objective is to utilize a physiologically relevant microreactor that has proved suitable for organotypic liver culture (3) to investigate cellular and molecular events during tumor metastatic seeding. The sub-millimeter scale of this liver allows for real-time imaging throughout the entire tissue over weeks in culture. We propose to use this system to determine when and how motility is rate-limiting for tumor progression. The first step in gaining this capability, and the one supported by the DoD funds is to determine whether an organotypic liver tissue culture supports metastatic establishment and growth.

## BODY

The revised and accepted Statement of Work (Table 1) described a series of tasks to accomplish the one accepted Objective. We have tackled these Tasks in the order of greatest yield so that work in areas can progress as systems are being optimized in others. The main efforts during the first year of this two-year project have been focused on the establishing system for the tumor cells. During the two year time frame of this grant we have accomplished these tasks.

*Table 1. Statement of Work*

**<u>Work to be performed at University of Pittsburgh (Wells and Stolz Laboratories):</u>**

1. isolate hepatocytes and endothelial cells
2. label tumor cells
3. seed bioreactors with cells
4. seed organotypic liver bioreactors with tumor cells

**<u>Work to be performed at MIT (Griffith and Lauffenburger Laboratories):</u>**

5. design bioreactor scaffolds for high volume production
6. optimize new bioreactor for continuous two-photon imaging
7. produce bioreactor scaffolds
8. deconvolute images to determine tumor-hepatocyte involvement and growth dynamics

~~*Work to be performed at University of Pittsburgh:*~~

*Task 1. Isolate hepatocytes and endothelial cells.* This task has been established. We have been successfully isolating both hepatocytes and endothelial cells as viable cells. These have been isolated both from wild-type rats and from GFP-expressing rats. These have been incorporated into the pre-bioreactor spheroids (Figure 1). We routinely obtain over 80% viability in these preparations. This is sufficient for generating the organotypic culture system.

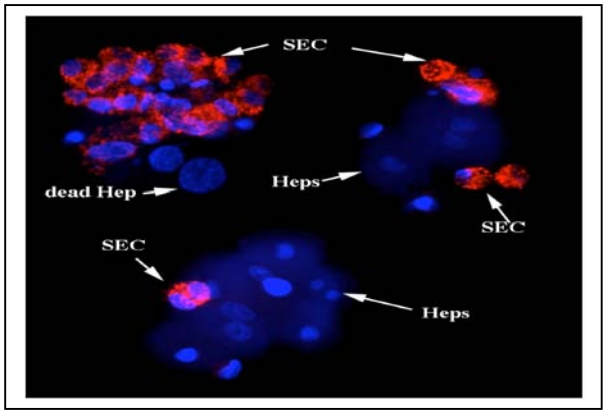



Figure1. The rat endothelial cells (vitally stained red by di-I uptake, and labelled SEC in figure) are alive within the hepatocyte spheroids (nuclei of all cells stained blue by DAPI; hepatocytes are identified by morphology and labelled heps) prior to loading of the bioreactor.

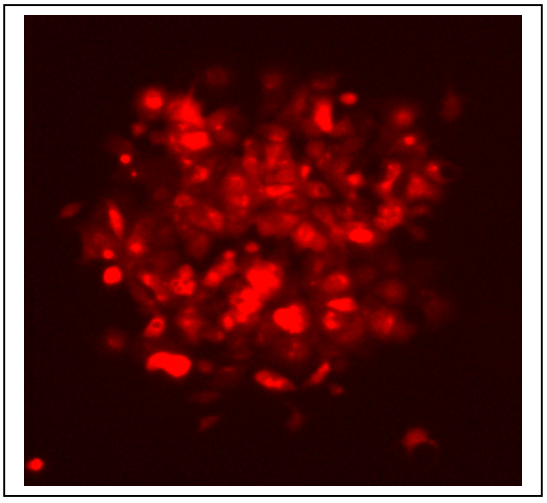


Figure 2. MCF7 breast cancer cells fluorescencing red from stable expression of RFP. RFP-expressing cells were selected by flow cytometry and passaged in culture for over 3 weeks. Virtually all cells express fluorescence demonstrating feasibility of following these cells for up to a month in the bioreactor.

*Task 2. Label tumor cells.* We established MCF7, MDA-468 and MDA-231 cells stably expressing RFP in addition to GFP (Figure 2a). In order to track primary breast cancer cells, we needed a method to label these cells. Stable transfection would not be consistent with maintaining these cells in early passage and heterogeneity. We used cell tracker dyes to label these cells.

*Task 3. Seed bioreactors with cells.* This task has been fully established. We have moved to generating liver bioreactors with transgenic hepatocytes and/or endothelial cells expressing GFP to better image the interactions with the tumor cells. These cells form bioreactor structures indistinguishable from non-transgenic cells (Figure 3). As the hepatocytes have an endogenous fluorescence in the same channel as GFP, this interfered with imaging tumor cells in this channel. As such, we have switched our tumor cell imaging to the red channel with RFP.

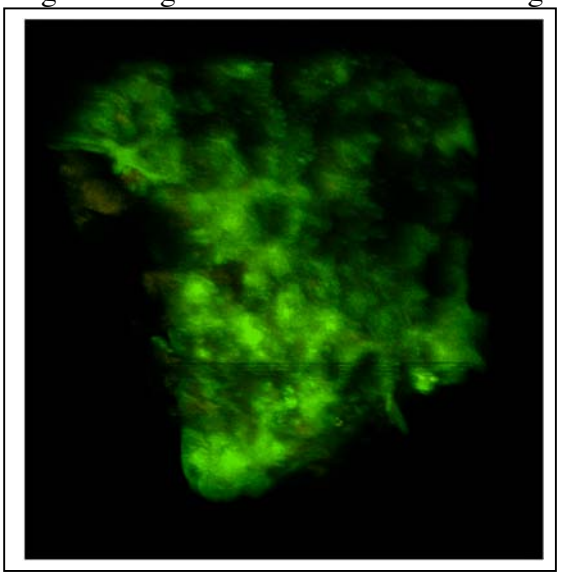


Figure 3. The hepatocytes and non-parenchymal cells from the GFP-transgenic rats establish channel-filled organoid cultures in the bioreactor similar to non-GFP-transgenic animal cells, as shown here 5 days after seeding.

*Task 4. Seed organotypic liver bioreactors with tumor cells.* This task is fully established. We have accomplished this task with breast tumor cell lines (Figure 4) and primary cells (Figure 5). The tumor cells proliferate over a few day period to eventually take over the entire bioreactor.

The newer bioreactor scaffolds that are higher volume production also enable better histological and electron microscopic examinations. However, seeding of tumor cells in these bioreactors requires different flow conditions. A posteriori, we have optimized the flow conditions to enable tumor cell seeding in these bioreactors. We will test the reproducibility and parallel processing of our 24 well bioreactor during our no cost extension.

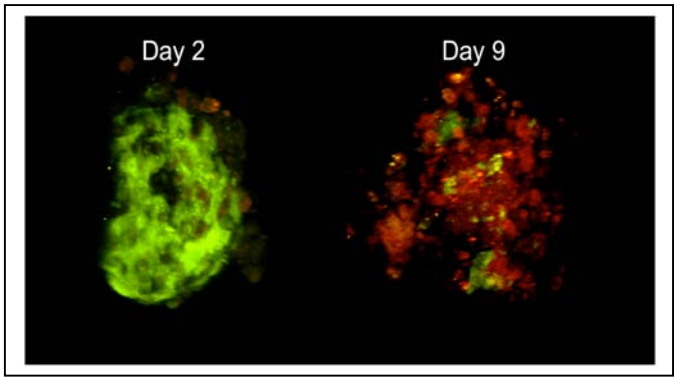



Figure 4. The RPF-labelled tumor cells expand from a few isolated cells (red) among the hepatocyes (green) noted 2 days after seeding the bioreactor (left image), to form the predominant cell mass a week later (right image). Shown are images from the identical bioreactor channel; these are representative of over 50 total channels in at least 6 independent experiments.

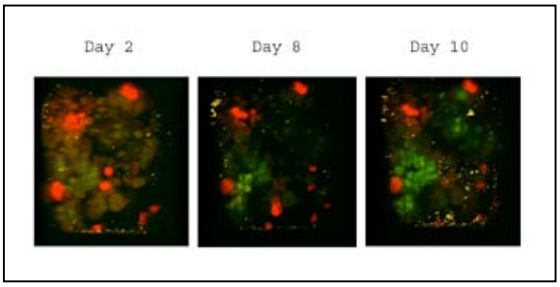
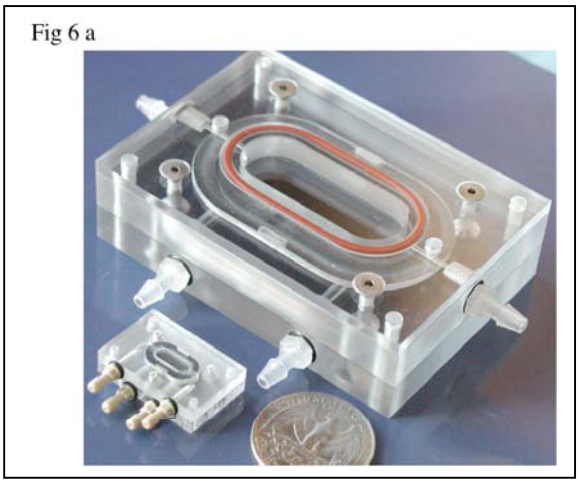


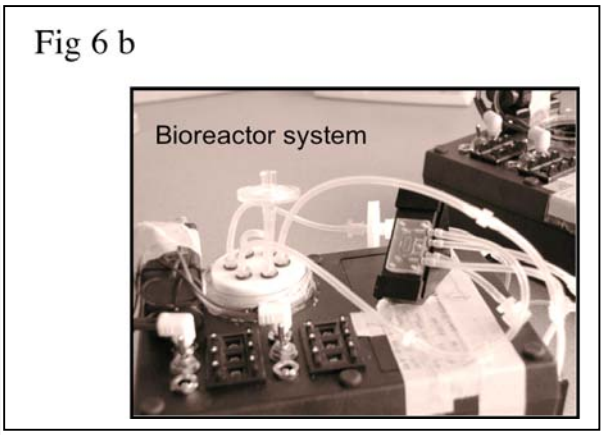

Figure 5. Primary breast cancer cells were obtained as a gift from Precision Therapeutics Inc. These were dyed with red cell tracker and inoculated into the liver organotypic bioreactor.

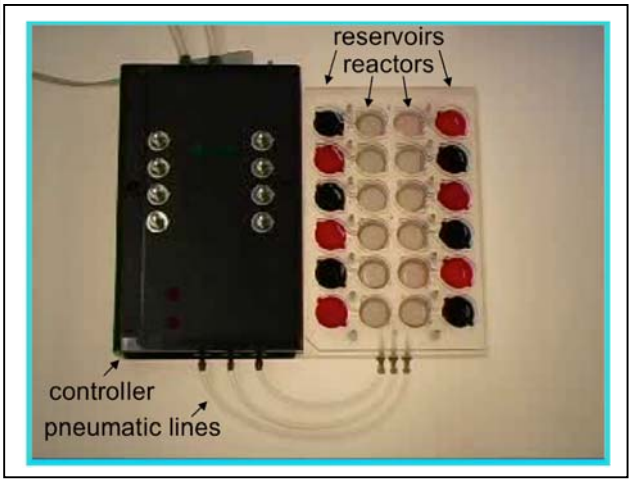
*Work to be performed at MIT:*

*Task 5. Design bioreactor scaffolds for high volume production.* The original bioreactor format was designed for in situ 2-photon imaging and requires a substantial technical effort to seed and run each individual reactor. This approach may be acceptable for 2-photon imaging, but there other assays of interest that are desirable to perform on a high-throughput basis in a multi-well format, including assays of cell growth rate, influence of various non-parenchymal cell types on tumor behavior, and histology. Thus, a multi-well plate format version of the bioreactor based on microfluidic pumping has been developed (Figure 6). The current prototype has 24 wells in a standard 24-well plate footprint and can be loaded with cells by simple pipetting. We are currently validating that the liver function in this system is comparable to that in the original reactor, using a panel of PCR probes for key P450 enzymes and liver-enriched transcription factors. These are the focus of our no cost extension.

Figure 6. Three version of the bioreactor are currently used for studies. (a) the MilliReactor, which holds 50,000 cells and the Giant reactor, which holds 1.3 million cells (b) The millireactor in its fluidic circuit, showing the pumps and medium reservoir and battery power (c) the multi-well plate reactor, which can be scaled to hold 10,000 – 1,000,000 cells per well. In (c), colored fluids have been added to the medium reservoir to illustrate the fluid path.




*Task 6. Optimize new bioreactor for continuous two-photon imaging*. We found that the imaging protocols have very specific demands on reactor construction. Although scaffolds made from plastic materials such as polycarbonate are preferable for histological sectioning, these scaffolds have properties that cause excessive heat generation during 2-photon imaging. We thus have determined that the 2-photon imagine experiments are best conducted with silicon scaffolds, but we have also identified fabrication methods for polymer scaffolds that will allow other experiments to be conducted in a high throughput format. The development of this bioreactor will be continued during our no cost extension.

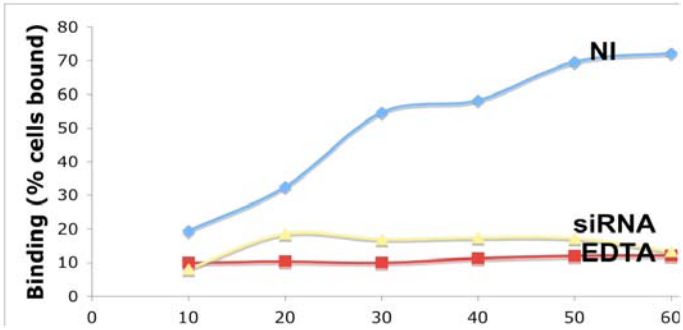
*Task 7. Produce bioreactors and scaffolds for U.Pitt research*. This is an ongoing task that generates sufficient bioreactors for studies. To-date, over 25 bioreactor scaffolds and assemblies have been delivered for use in these studies outlined herein. We fabricate silicon scaffolds using deep reactive silicon etching at MIT, and produce these on a semi-high throughput basis. We investigated several approaches to fabricating scaffolds from polymers to facilitate histological sectioning and to use in the multi-well plate format of the system. Approaches included laser machining of polycarbonate, polystyrene and polyimide sheets, injection molding of polypropylene, and micromachining of polycarbonate. Among these methods, micromachining of polycarbonate waA the most promising, and we currently use these reproducible surface properties. These will be made during our no cost extension.

*Task 8. Deconvolute images to determine tumor-hepatocyte involvement and growth dynamics.* The images of breast cancer cells in the bioreactor have been deconvoluted in four dimensions (through time). The cells have been seen to intercalate between the hepatocytes in the liver microtissue (Figure 5).

*Additional task*

Based on preliminary finding of breast cancer cells juxtaposing to hepatocytes (Figure 7), we determined whether such cells can adhere to hepatocytes via E-cadherin homotypic binding. This is listed as a change in the Year 1 report which was approved. This has been accomplished for MCF7 cells which express E-cadherin (Figure 7). MCF7 bound to hepatocytes in a E-cadherin-dependent manner as EDTA, which chelates calcium eliminating E-cadherin homotypic binding, and siRNA to E-cadherin eliminated this association.

Figure 7. MCF-7 breast cancer cells that express E-cadherin form heterotypic binding to a monolayer of hepatocytes as determined by centrifugal assay for cell adhesion (CAFCA) (Nl). This is due to E-cadherin as it is disrupted by the calcium chelator EDTA (EDTA) and siRNA that downregulates E-cadherin by >70% (siRNA).




## KEY RESEARCH ACCOMPLISHMENTS

- MCF7 breast cancer cells stably expressing GFP and RFP
- MDA-468 and MDA-231 cells stably expressing RFP
- Labelled primary breast cancer cells with red cell tracker dye
- Routine isolation of viable hepatocytes and liver endothelial cells
- Routine establishment of mixed hepatocyte and endothelial cell bioreactors
- Production of higher volume bioreactor scaffolds
- Production of 25 bioreactor scaffolds and set ups for experimentation
- Optimization of tumor cell seeding protocol
- Growth of breast tumor cells in the bioreactor
- Proof-of-principle that breast cancer cells bind to hepatocytes via E-cadherin

## REPORTABLE OUTCOMES

*Articles:*

CC Yates, CR Shepard, G Papworth, **DB Stolz**, S Tannebaum, **L Griffith**, **A Wells** (2006). Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Advances in Cancer Research, in press.

*Abstracts:*

C Yates, **D B Stolz, L Griffith, A Wells** (2004) Direct Visualization of Prostate Cancer Progression Utilizing a Bioreactor. American Association for Cancer Research Annual Meeting, Orlando, FL oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Ex Vivo Metastasis Model for Prostate Cancer Progression. Regenerate 2005, Altanta, GA oral presentation

**L Grififfth,** C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2005)

Microscale Tissue Engineering of Liver, 20th Aspen Cancer Conference, Aspen, CO oral presentation

A Dash, **L Griffith, A Wells,** S R Tannebaum (2005) Activation of c-Met in Hepatocyte-Prostate Cancer Cell Co-cutlures, 20th Aspen Cancer Conference, Aspen, CO poster presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Characterization of Prostate Cancer Progression by Direct Visualization Utilizing a Bioreactor. American Association for Cancer Research Annual Meeting, Anaheim, CA oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) Direct Visualization of Prostate Cancer Progression Utilizing an Organotypic Liver Bioreactor. Academy of Clinical Laboratory Physicians and Scientists Annual Meeting, Pittsburgh, PA oral presentation

C Yates, **D B Stolz, L Griffith, A Wells** (2005) An Organotypic Liver Bioreactor as a Metastasis Model for Tumor Progression. Regenerate 2005, Atlanta, GA poster

**L Grififfth,** C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2005) In Vitro Physiological Tissue Models for Drug Discovery, Emory/Georgia Tech Research Institute for Predictive Health, Predictive Health Symposium oral presentation

**L Grififfth,** C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2006) Human Body on A Chip: Microscale Tissue Engineering for Drug Discovery, New York Academy of Sciences, Predictive Toxicology Symposium oral presentation

**L Grififfth,** C Yates, A Sivaraman, K Domansky, **D B Stolz,** S R Tannenbaum, **A Wells** (2006) Human Body on A Chip: Microscale Tissue Engineering of Liver and Bone Marrow for Drug Discovery, Boston Discovery Toxicology Meeting, Cambridge, MA oral presentation

C Yates, C Shepard, **A Wells** (2006) A Critical role of E-cadherin expression during the Progression of Metastatic Prostate Cancer cells with the Microenvironment as a Driving Force. American Association for Cancer Research Annual Meeting, Washington, DC oral presentation

*Book chapters:*

C Yates, **DB Stolz, LG Griffith** (2005) Imaging Invasion and Metastasis ex vivo. In Cell Motility in Cancer Invasion and Metastasis (Ed: A Wells, Kluwer Academic Press).

**A Wells** (2005). Motility in tumor invasion and metastasis – an overview. In Cell Motility in Cancer Invasion and Metastasis (Ed: A Wells, Kluwer Academic Press).

## CONCLUSIONS

This award has reached defined milestones including the additional task added after Year 1. The systems are firmly in place to utilize this metastasis model system for mechanistic investigations in breast cancer. The establishment of this model has also highlighted new directions for future research in that we find close contacts between hepatocytes and tumor cells.

*Importance/Implications:* The Key Accomplishments above firmly demonstrate the ability to establish the model system. This provides the 'proof a concept' that such a model system can examine intermediary scale events during metastatic growth. Further, the finding of close heterotypic cell interactions highlights new avenues for study.

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2. J Condeelis, J E Segall (2003). Intravital imaging of cell movement in tumours. *Nature Reviews – Cancer* 3:921-930; J S Condeelis, J Wyckoff, J E Segall (2000). Imaging of cancer invasion and metastasis using green fluorescent protein. *European Journal of Cancer* 36:1671-1680; K J Luzzi, I C MacDonald, et al (1998). Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *American Journal of Pathology* 153:865-873
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